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Experimental Research

Kinetics of the cellular immune response following closed head injury

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Summary

Background. The contribution of brain edema to brain swelling in cases of traumatic brain injury (TBI) remains a critical problem. We believe that inflammatory reactions may play a fundamental role in brain swelling following a head injury. Although possible roles of microglia activation and the release of mediators have been suggested, direct evidence of cellular immune reactivity in diffuse brain injury following *closed* head trauma is lacking. Accordingly, the objective of this study was to assess the temporal pattern of microglia activation and lymphocyte migration in an experimental model of TBI.

Method. An impact acceleration TBI model was utilized to induce diffuse brain damage in adult Wistar rats. The animals were separated into three groups: unoperated controls, sham-operated controls and trauma group. At various times after TBI induction (5 min–24 h), rats were perfused transcardially. Sagittal brain sections were analyzed with immunohistochemical markers of CD3 to reveal the presence of T-lymphocytes, and by immunochemistry for the detection of CD11b to reveal microglia activation within the brain parenchyma.

Findings. In the control groups, scattered T-cells were found in the brain parenchyma.

In the trauma group, TBI induced microglia activation and a transient biphasic T-cell infiltration of the brain parenchyma in all regions was found, beginning as early as 30 min post injury and reaching its maximum values at 45 min and 3 h after trauma induction.

Conclusion. These results lead us to suggest that the acute response to severe head trauma with early edema formation is likely to be associated with inflammatory events which might be triggered by activated microglia and infiltrating lymphocytes. It is difficult to overestimate the clinical significance of these observations, as the early and targeted treatment of patients with severe head injuries with immunosuppressive medication may result in a far more favorable outcome.

Keywords: Closed head injury; lymphocytes; microglia; inflammation; rat.

Abbreviations

TBI Traumatic brain injury; *ICP* intracranial pressure; *BBB* blood–brain barrier; *CNS* central nervous system; *CHI* closed head injury; *MHC* major histocompatibility complex; *MRI* magnetic resonance imaging.

Introduction

An elevated intracranial pressure (ICP) subsequent to brain swelling is the single most frequent cause of death in head injured patients. Analysis by the American Traumatic Coma Data Bank indicates that the probability of mortality and morbidity increases with time during elevated ICP levels above 20 mmHg [31]. Thus raised ICP therefore continues to be a prominent feature of severe traumatic head injury. Morphological and magnetic resonance studies of traumatic brain injury (TBI) have provided compelling evidence that brain swelling

is associated with axonal injury and a predominantly cellular edema formation, which begins within 1 h post injury and becomes dominant at 1–2 weeks post injury [2, 33, 34, 36, 39]. Although information concerning the process of cytotoxic damage in TBI is limited [5, 6], there is some evidence of an inflammatory response in the pathogenesis of head injury; as such, brain swelling appears to be, in part, an inflammatory process [9, 11, 22, 29].

The central nervous system (CNS) has often been considered an "immunologically privileged site", this presumption being based on the absence of lymphatic drainage and the unique type of endothelium forming the blood–brain barrier (BBB). The data suggest that, even under normal conditions, there is a moderate traffic of hematogenous cells through the BBB [3, 18, 42]. In immune-mediated illnesses of the CNS, large quantity of the cells of the immune system is permitted to enter the brain [15, 16, 27, 35, 41]. These inflammatory cells capable of entering the CNS include T-cells and macrophages, whereas B-lymphocytes are less abundant and neutrophils are rarely detected.

There are two major subtypes of TBI: focal and diffuse damage. It is clear that cortical contusion, and particularly a penetrating head injury such as a stab wound damaging the BBB, leads to the recruitment of numerous circulating monocytes and white blood cells [19, 20]. Since contamination of the contusion in open head injuries clearly visible, the contribution of inflammation to traumatic brain swelling in such injuries has never been questioned. Although potential roles of microglia activation and the release of mediators have been suggested, direct evidence of the cellular immune reactivity (and entry of immune cells into the brain) in diffuse TBI following induction of a closed head trauma has not been presented so far. Furthermore several papers including Csuka's *et al.* proved that the contusional model elicits an entirely different immunological reaction pattern than does the diffuse brain injury model [8].

In light of this, our aim was to assess the time-dependent changes in microglia activation and lymphocyte migration in an experimental model of diffuse TBI. With this aim, our first step was to provide evidence of the entry of lymphocytes into the brain; next, we attempted to describe the temporal characteristics of lymphocyte migration.

Materials and methods

One-hundred adult male Wistar rats (weighing 320–350 g) were used in the study. The experiments were conducted in accordance with prevailing laws and ethical considerations. Written permission was obtained in advance from the University of Szeged Faculty Ethical Committee on Animal Experiments. The rats were initially anesthetized with isoflurane, then intubated and artificially ventilated with a gas mixture of N_2O (70%), O_2 (30%) and isoflurane (0.5–1.5%). The rectal temperature was monitored and the body temperature was maintained at $37 \pm 0.5\,^{\circ}C$ by means of heat.

Impact acceleration injury

An impact acceleration head injury model was used to produce trauma [33]. A midline scalp incision was made, the skin and periosteum were reflected, and the skull was carefully dried. A round stainless steel disc was mounted on the skull with super glue. When the bonding agent was dry, the rat was positioned under a hollow Plexiglas tube, disconnected from the respirator, and a sectioned brass weight of 450 g was dropped from a height of 2 m onto the center of the metal disc. Under these experimental conditions, a mortality rate of 44% resulted with a low incidence of skull fracture [33]. The disc was used with a view to preventing skull fracture. After induction of the trauma, the rat was rapidly reconnected to anesthesia and artificially ventilated, and the wound was closed.

Schedule of the measurements

The rats ($n = 100$) were separated into three groups: *group Ia:* unoperated controls ($n = 20$); g*roup Ib:* sham-operated controls ($n = 20$); g*roup II:* trauma ($n = 60$).

At various times after TBI induction (5, 15, 30 or 45 min, or 1, 2, 3, 6, 12 or 24 h), the rats (6 at each survival time) were perfused transcardially with 500 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

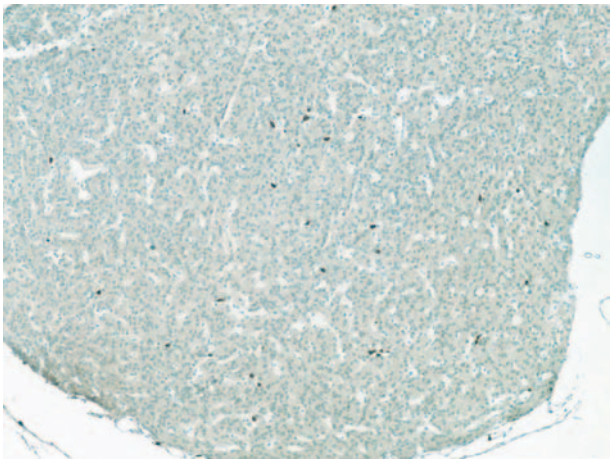

Fig. 1. Photomicrograph of CD3-positive T-lymphocytes in rat pineal gland, control group (×10)

CD3 (T-lymphocyte) immunohistochemistry

Postfixed brains were routinely embedded in paraffin and 4 µm thick serial sagittal sections were cut and air-dried on silanized slides. For the immunohistochemistry of CD3, the samples were dewaxed in xylene, and rehydrated in a series of decreasing concentrations of ethanol. The sections were submerged in a target retrieval solution of DAKO (code no. S1699), and boiled in a pressure-cooker for 5 min.

The specimens were pre-incubated in 10% normal swine serum, and processed for immunohistochemistry, using rabbit anti-human CD3 (DAKO; code no. N1580; prediluted form) overnight at room temperature. The sections were then reacted with DAKO EnVision+ (EnVision+, Peroxidase, Rabbit; code no. K4003) for 1 h. The immunereactions were developed in 3,3′-diaminobenzidine (Sigma) for 30 min, then slightly counterstained with hematoxylin (DAKO Automation Hematoxylin; code no. S3301), and finally covered with DePeX (Fluka).

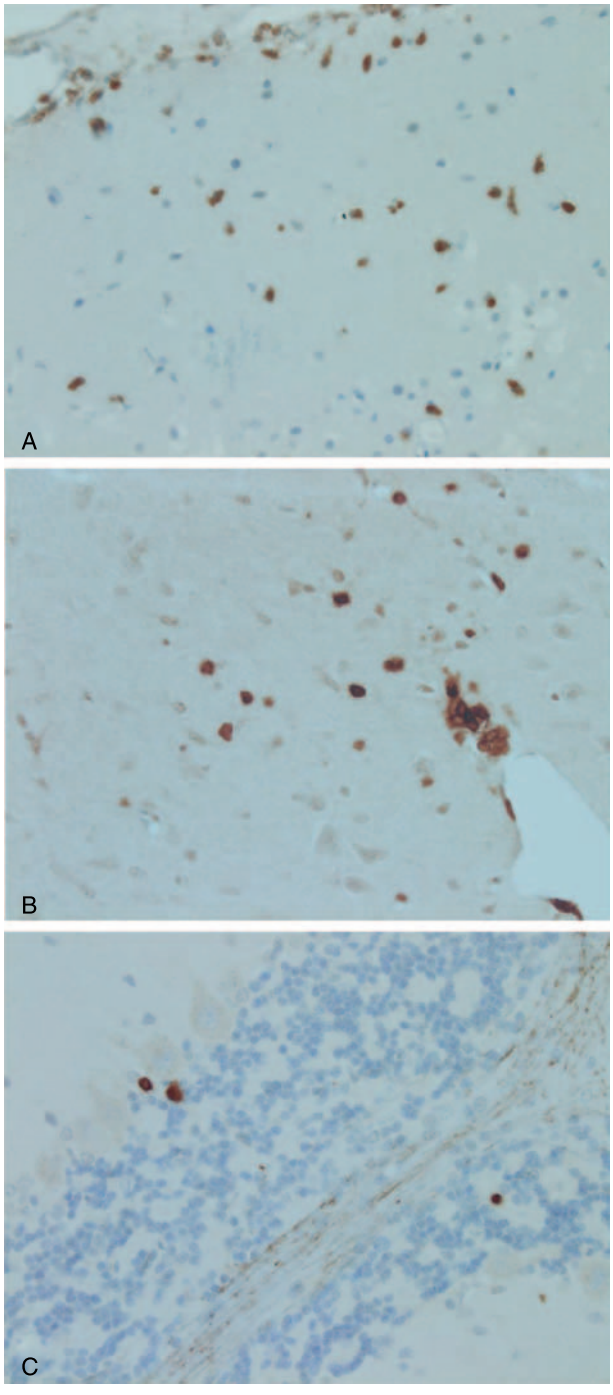

Fig. 2. Photomicrographs of CD3-immunoreactive T-lymphocytes in rat neocortex (A), brain stem (B) and cerebellum (C) (×40) 2 h after closed head injury induction

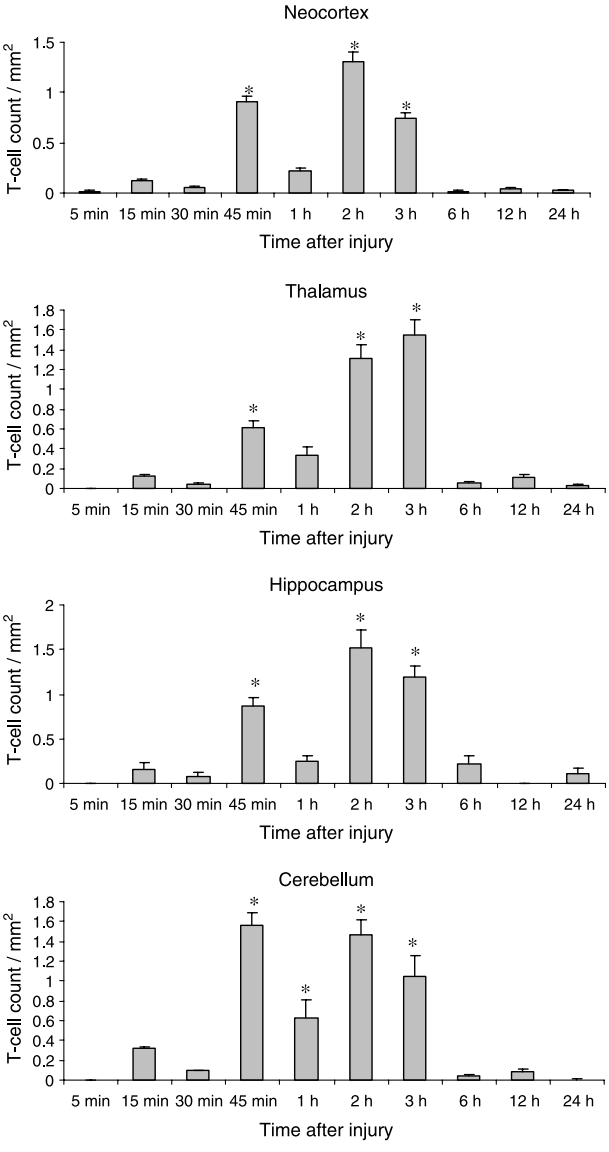

Fig. 3. Kinetics of presence of CD3-immunoreactive T-lymphocytes in different brain regions. Values shown means ± standard deviation (*SD*), $^{*}p < 0.001$, as compared to control or value at 5 min

Sections from the brain were analyzed semiquantitatively in each group. Ten sections from each brain were examined and the T-cells in the brain parenchyma were counted under a light microscope (E600; Nikon). The brain regions examined included the cortex, diencephalon, brain stem and cerebellum. The numbers of T-lymphocytes were examined by means of repeated measurement ANOVA.

CD11b (microglia) immunohistochemistry

The brains were dissected and postfixed for 16 h at 4 °C. Following postfixation, the brains were cryoprotected overnight in 30% sucrose-containing 1 M phosphate buffer, pH 7.4. The brain samples were cut at coronal planes on a freezing microtome. Serial sections of 24 µm thickness were collected in 0.01 M phosphate buffer containing 0.9% NaCl, supplemented with 0.1% sodium azide. Representative free-floating sections were pretreated in 3% H_2O_2 and in 0.5% Triton X-100 for 10 min each. The samples were treated in 10% normal swine serum for 1 h, and then processed for immunohistochemistry, using biotin-labelled mouse anti-rat CD11b antibody (Serotec; cat. no. MCA275B) at a dilution of 1000 at room temperature overnight. Next, specimens were incubated in a 1/1000 dilution of peroxidase-labelled streptavidin (Jackson Immunoresearch) for 1 h. Primary antibody binding was visualized with 3,3′-diaminobenzidine (Sigma) in the presence of nickel ammonium sulphate for 15 min. The sections were finally mounted on glass slides, air-dried and covered with DePeX (Fluka).

Statistical analysis

The mean OD values were calculated; the data for the statistical analysis were analyzed by means of SPSS v9 computer programme. The numbers of T-lymphocytes were examined by means of repeated measurement ANOVA. The control and injured animals were compared by one-way ANOVA (post-hoc test: Tukey method).

Results

T-cell entry into the CNS parenchyma

Control groups (Ia and Ib): In sections showing CD3 positivity, most T-lymphocytes were found in regions without a BBB such as the area postrema and the pineal gland (Fig. 1). A few T-cells (0–0.05/mm^2) were also found in the brain parenchyma with an intact BBB, but this finding appeared consistent during the next 24 h.

Trauma group (II)

As expected, the number of infiltrating lymphocytes did not reveal any change in regions with a BBB during the first 30 min after TBI induction.

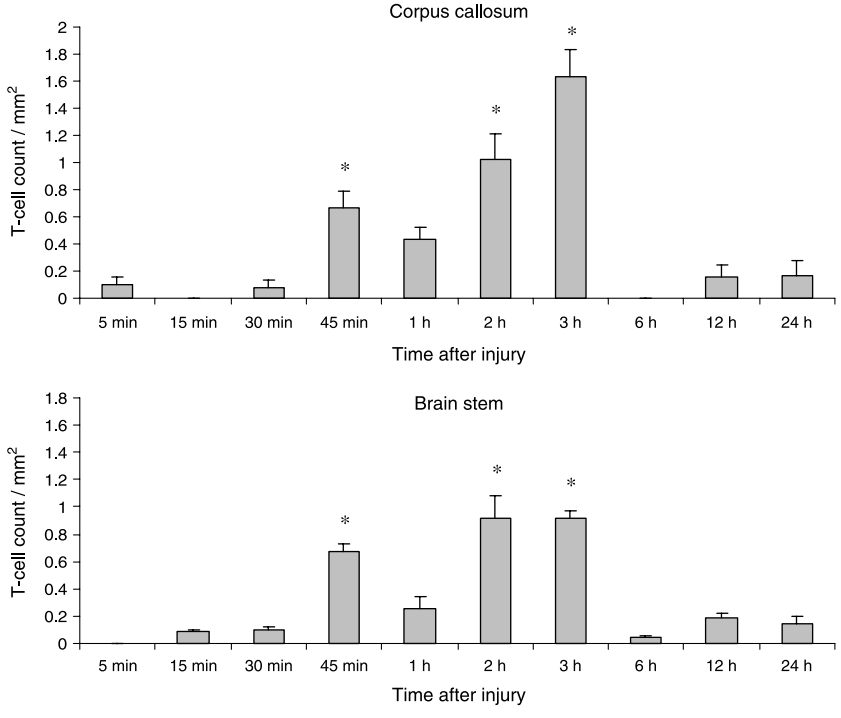

Fig. 4. Kinetics of presence of CD3-immunoreactive T-lymphocytes in white matter of rat brain. Values shown means ± standard deviation (*SD*), $^*p < 0.001$, as compared to control or value at 5 min

Over the next few hours, however, the TBI-induced T-cell infiltration displayed a biphasic pathophysiological response. The number of CD3-positive T-lymphocytes started to increase at 30 min post trauma and reached a maximum level at 45 min post injury. After a temporary decrease at 60 min, the number of CD3-positive T-lymphocytes again began to increase, reaching its peak level (exceeding that observed at 45 min post-injury) at 2–3 h post injury, depending on the brain region examined (Fig. 2).

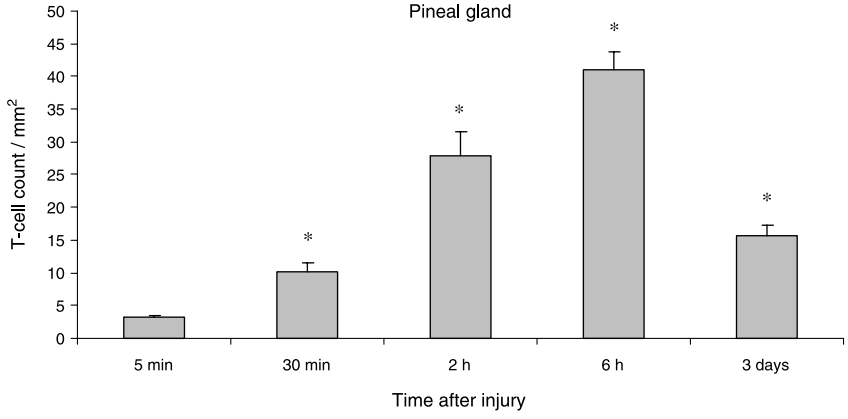

Fig. 5. Kinetics of presence of CD3-immunoreactive T-lymphocytes in rat pineal gland. Values shown means ± standard deviation (*SD*), $^{*}p < 0.001$, as compared to control or value at 5 min

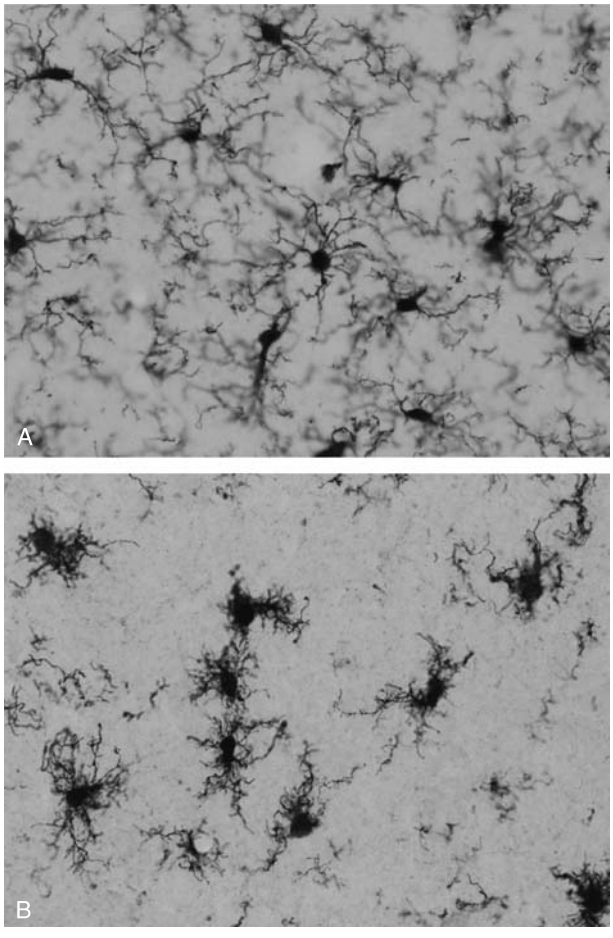

Fig. 6. Photomicrographs of CD11b-immunoreactive microglia in rat cerebral cortex. (A) Resting microglia in neocortex. (B) Activated ramified microglia in neocortex 1 h after closed head injury induction (×60)

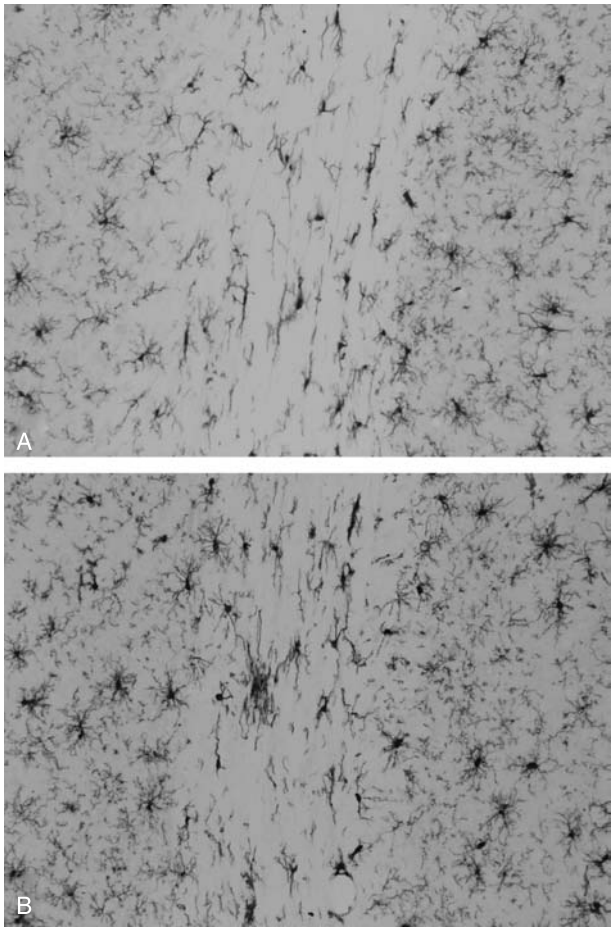

Fig. 7. Photomicrographs of CD11b-immunoreactive microglia in white matter. (A) Resting microglia in corpus callosum. (B) Activated ramified microglia in corpus callosum 1 h after closed head injury induction (×20)

During the next several hours, the number of T-cells decreased rapidly; at 24 h post injury no significant difference was observed in any region as compared with the controls (Fig. 3).

The white matter in the cerebrum, cerebellum and the brain stem displayed the highest T-cell concentration ($1–2/mm^2$) (Figs. 3 and 4).

In regions without a BBB (pineal gland and area postrema), the number of T-cells gradually increased to its peak value ($40–45$ cell$/mm^2$) at 6 h post injury (Fig. 5).

Microglia activation

Coronal brain sections labelled with anti-CD11b antibodies were analyzed for microglia activation. One hour after TBI induction, the microglia had proliferated and displayed a rapid transformation from a resting to an activated state. During this time, the cell body became hypertrophic, with long, branched and crenellated processes. In the course of the next few hours, the shape of the microglial cells changed again, with a morphological transformation from the previous bipolar/ramified form into an activated amoeboid form. This amoeboid shape remained unchanged during the next 24 h (Figs. 6 and 7).

Discussion

Induction of an inflammatory process in the CNS has been reported following brain trauma, but no study has been performed to assess the cellular immune reactivity in a *closed* TBI so far. The present study revealed that severe diffuse TBI induces microglia activation and a transient biphasic T-cell infiltration of the brain parenchyma in all regions, beginning at 30 min post injury and peaking at 45 min and at 3 h after trauma induction. Despite the diffuse nature of the injury model applied, marked differences were detected between the various brain regions analyzed; the highest alterations in activity were restricted to the white matter of the cerebrum, the cerebellum and the brain stem.

T-cell survey of the brain

Until recently, little consideration was given to inflammation as a significant factor in the pathophysiology of secondary brain damage associated with *closed* head injury. The presence of astrocytes and microglia that embody the immune function, and the restrictive BBB, which is also known to possess immune functions, argue strongly for the unique immune status of the CNS [13, 42]. It is now clear that the CNS is characterized by only a partial immune privilege, in terms of downregulation and suppression of many aspects of the immune function in comparison with other organs. Almost all tissues contain strategically distributed antigen-presenting cells that constitutively express major histocompatibility complex (MHC) antigens and play a primary role in the initiation of an immune response. The concept of immune surveillance has been confirmed for almost all vertebrate tissues, with the noteworthy exception of the CNS. The immune status of the brain, however, has been re-evaluated recently, in order to learn more about the cellular auto-immune reactions that are suspected of playing a key role in the pathogenesis of a number of autoimmune diseases in the CNS [4, 42]. Experiments with T-cell lines specific for CNS antigens have led to the surprising conclusions that the CNS is routinely surveyed by activated T-lymphocytes that can cross the BBB, and that astrocytes play a major part in the initiation and subsequent regulation of the intracerebral immune response [17, 30, 42]. These results are in accordance with our findings of a significant number of T-lymphocytes in the control group in regions without a BBB, and a few T-cells even in those regions which are protected by the BBB. Although the number of these T-cells appeared very low ($0–0.05$ cell$/mm^2$), the antigen recognition by these lymphocytes following a head injury might be crucial in terms of triggering the ensuing immune response.

Dynamics of lymphocyte migration

In this diffuse head injury model, we have found a transient biphasic T-cell infiltration of the brain parenchyma in all regions, which begins 30 min post injury and reaches its maximum at 45 min and at 3 h after trauma induction, indicating two different mechanisms of lymphocyte migration. In a previous study, in which the BBB was tested with a contrast agent (Gd-DTPA) in MRI, we demonstrated that the BBB opens at the time of the trauma and approaches closure at about 60 min post injury [1]. On the basis of these findings, we presume that the first surge of T-cell infiltration might be consistent with the BBB disruption, while the second and more pronounced surge at 3 h post injury could be a result of T-cell activation. Since the BBB opening is transient, some of the lymphocytes might be trapped, while the others (circulating-T cells in the brain vessels) might not be able to penetrate the rapidly re-established BBB. This could be the explanation of the temporary decrease in T-cell number 1 h post injury.

The large number of T-cells seen in all brain regions during the second surge (at 3 h following TBI induction) indicates the presence of specific targeting of the immune response, supporting specific connections between the CNS and the immune system. Although these surveying lymphocytes and the T-cell infiltration demonstrated during BBB disruption in our study are sufficient to produce activated T-lymphocytes, other features might also contribute to the further development of the cellular immune response. By what mechanism the T-cell activation is induced is still not fully understood, but the availability of T-cell lines specific for CNS antigens raises the fundamental question of which cell type(s) can present the antigen (myelin basic protein?) in order to further activate the T-cells once they are within the CNS. There are two possible ways in which antigens can be processed and presented to the immune system by the CNS. First, by the two major antigen-presenting cell types: microglia and perivascular cells. However, these cells are remote from regional lymph nodes and few of them can trigger the activation of circulating lymphocytes in the CNS. Alternatively, antigens could be presented to lymphocytes in regional lymph nodes by the drainage of antigens themselves or by the migration of antigen-presenting cells [4, 29, 43]. It has recently been shown that not only are there pathways for the lymphatic drainage of interstitial fluid from the brain, but also the drainage of such proteins exerts a significant effect upon antibody production within the regional lymph nodes in the neck [24, 25].

Since our investigations targeted at the cellular immune response were restricted to the first 24 h we could only assess the acute cellular response. Although the 3 h period of immune cell invasion demonstrated in our study is sufficient to trigger a cascade of events resulting in tissue specific inflammation and significant brain edema, other features, such as delayed BBB opening and astrocytic swelling might also contribute to the late edema, which begins a few hours post injury and exhibits a maximum effect between 3 and 8 days after trauma induction [1, 34]. Further studies are necessary to determine the applicability of these factors to diffuse injury.

Time-dependent microglia activation and interaction between lymphocytes and microglia

The inflammation associated with CNS injury involves two major components: the activation of intrinsic microglia cells and the recruitment of bone marrow-derived inflammatory cells from the peripheral bloodstream [37, 40].

It is generally accepted that both microglia and peripheral monocytes respond to injury in various proportions, depending on the type and severity of the lesion. Chemical injuries to the brain appear to lead to a predominantly microglia cell inflammatory response, while direct stab wound injuries (with BBB disruption) involve mostly peripheral monocytes.

In our closed head injury model, we discerned a rapid transformation of the microglia; the cell body became hypertrophic, with long, branched and crenellated processes during the first 60 min post injury. Although we focused exclusively on the microglia activation, the short duration of BBB damage and the microglia transformation observed within 60 min post injury suggest that predominantly microglia cells contribute to the induction of the immune response, while peripheral monocytes possibly play a role only in the eventual development of cystic cavities after the clearance of damaged tissue. This lends support to the report of microglia activation within 1 h in a similar scenario, followed considerably later by macrophage and neutrophil infiltration in spinal cord, peripheral nerve and brain injury [8, 10, 23, 26, 38].

Lassmann *et al.* found both microglia cell processes and the soma of these cells in the perivascular astrocyte layer of the glia limitans reaching, the vascular basement membrane of the BBB [28]. This microglia processes express class I and II MHC antigens, while the astrocytic processes remain unstained by these MHC-specific antibodies. Lassmann *et al.* were able to demonstrate microglia processes in 4–13% of all vessel cross-sections, irrespective of their size and the type (artery, capillary or vein) of the vessel segment [28]. The presence of these immunocompetent microglia cell processes in the glia limitans, with the potential to function as antigen-presenting elements, could account for our observation that lymphocyte infiltration develops in the brain parenchyma 3 h following a closed head injury. Since microglia cells have been shown to produce inflammatory mediators (cytokines), acting as antigen-presenting cells for T-lymphocytes, our present observations of early microglia activation and lymphocyte infiltration further support the premise that these cells may play a fundamental role in the induction and maintenance of the inflammatory process in the CNS following a closed head injury [7, 12, 14]. On the other hand, while T-lympocytes and activated microglia may be key to the ongoing neurodegenerative processes, they may also equally be

secondary to other processes, such as excitotoxicity and related mechanisms.

Conclusions

A diffuse closed head injury induces T-cell infiltration of the brain parenchyma and rapid microglia transformation in all regions, which begins 30 min post injury and peaks between 45 min and 3 h after trauma induction.

These results lead us to suggest that the acute response to severe head trauma with early edema formation is likely to be associated with inflammatory events which might be triggered by activated microglia and infiltrating lymphocytes.

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